

## Original Article

# Down-regulation of miR-181b promotes apoptosis by targeting CYLD in thyroid papillary cancer

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**Abstract:** MicroRNAs (miRNAs) are a small class of non-coding RNAs that are widely deregulated in various cancers. They act as either oncogenes or tumor suppressor genes in human cancer. The purpose of this study was to examine the potential role of miR-181b in human thyroid papillary cancer. The expression levels of different miRNAs were measured by micro array analysis in 10 thyroid papillary cancer specimens and adjacent normal thyroid cancer tissues. MTT assays, colony formation assays, apoptosis assays were used to explore the potential function of miR-181b inhibitor in TPC1 human thyroid papillary cancer cells. Luciferase reporter assays were performed to validate the regulation of a putative target of miR-181b, in corroboration with qPCR and western blot assays. We found that the expression of miR-181b was higher in thyroid papillary cancer specimens compared with adjacent normal tissues ( $P < 0.05$ ). Downregulation of miR-181b inhibited cellular growth and promoted cellular apoptosis. Luciferase assays indicated that miR-181b can bind with its putative target site in the 3'-untranslated region (3'-UTR) of CYLD, suggesting that CYLD is a direct target of miR-181b. Western blot analysis indicated that downregulation of miR-181b results in the upregulation of CYLD at protein levels. Taken together, downregulation of miR-181b expression causes cellular growth inhibition, promoting cellular apoptosis by targeting CYLD. These findings suggest that downregulation of the expression of miR-181b may be a therapeutic target for the treatment of human thyroid papillary cancer.

**Keywords:** MiR-181b inhibitor, thyroid papillary cancer, CYLD

## Introduction

Papillary thyroid carcinoma (PTC) is the most common pathological type of thyroid malignant tumor, and at least 80% of all thyroid cancers are PTC [1]. Most of the PTCs have a good prognosis by surgical resection combined with radio iodine and levothyroxine treatment, while sometimes, metastasis and recurrence still happened in certain PTCs [2]. Certain clinical and pathological characteristics have been associated with a poor prognosis, such as advanced age at diagnosis, larger primary tumor ( $\geq 3$  cm), extrathyroidal invasion, lymph node metastasis and advanced tumor-node-metastasis (TNM) stage [3].

MicroRNAs (miRNAs) are endogenous single-stranded non-coding RNAs of about 22 nucleotides that interacting preferentially with the

3'-untranslated regions (3'-UTRs) of target mRNAs [4]. MiRNAs may regulate thousands of human genes and have fundamental importance in biological processes such as cell differentiation, proliferation, and survival [5]. Accumulating evidence suggests that miRNAs play essential roles in the regulation of tumor cell proliferation, differentiation, apoptosis and metastasis [6]. In recent years, extensive investigations of miRNAs have demonstrated critical roles of miRNAs in the development and progression of cancer, and some miRNAs are proposed as novel potential targets for cancer therapy [7]. Previous studies showed that miR-181b has played a role to regulate cellular growth, migration, invasion and apoptosis in several kinds of tumors, such as gastric adenocarcinomas [8], chronic lymphocytic leukemia [9], ovarian cancer [10], cervical cancer [11]. Some studies suggested that the expression of

miR-181b was significantly higher in thyroid PTCs compared with normal thyroid tissue [12, 13] and our experiment of microRNA-array analysis also demonstrated that miR-181b was higher in thyroid PTCs, but until now, the mechanism of miR-181b in PTC has been unclear.

The NF- $\kappa$ B pathway has an important role in the regulation of inflammatory responses that are linked to tumorigenesis [14]. Increased NF- $\kappa$ B activation in thyroid cancer cell lines and tissues has long been documented [15] and recent studies demonstrate that the NF- $\kappa$ B pathway controls proliferative and anti-apoptotic signalling pathways in thyroid cancer cells [16]. CYLD, a K63-specific deubiquitinase, has been demonstrated to negatively regulate NF- $\kappa$ B activity, inhibit the NF- $\kappa$ B signal transmission by specifically deconjugating the K63-linked polyubiquitin chains from multiple intermediaries of the NF- $\kappa$ B signaling pathway, such as TRAF2, TRAF6, RIP1, NEMO and BCL3 [17, 18].

In this study, we demonstrated that downregulation of miR-181b inhibited cellular growth, promoted cellular apoptosis, likely by targeting CYLD. The results indicate that downregulation of miR-181b level may be a therapeutic target for the development of future treatments of thyroid cancer.

### Materials and methods

#### *Specimens*

This study is approved by Institutional Ethics Committees of Tongji University (the approval number: SHSY-IEC-pap3.0/13-3). Ten paired thyroid papillary cancer specimens and adjacent normal thyroid papillary tissues were collected from the Department of General Surgery of the Shanghai Tenth People's Hospital. The samples were immediately snap-frozen in liquid nitrogen. All samples were confirmed as thyroid papillary cancer by trained pathologists. No patients received chemotherapy or radiotherapy prior to surgery.

#### *MicroRNA array analysis*

The 10 pairs of thyroid cancer tissues and para-cancer tissues were handled using TRIzol (Invitrogen) and miRNeasy mini kit (QIAGEN) according to the manufacturer's instructions. Total RNA (10  $\mu$ g) was size fractionated (< 200 nucleotides) by using a mirVana kit (Ambion Inc., Austin, TX) and labeled using the miR-

CURY™ Hy3™/Hy5™ Power labeling kit and hybridized on the miRCURY™ LNA Array (v.16.0). The slides were scanned using the Axon GenePix 4000B microarray scanner. Scanned images were then imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. Data adjustments included data filtering, log<sub>2</sub> transformation, and gene centering and normalization. The t-test analysis was conducted between thyroid cancer tissues and para-cancer tissues, and miRNA with *P* values < 0.05 were considered statistically significant.

#### *Cell culture and transfection*

Human Nthy-ori 3-1 normal thyroid follicular epithelial cells, human TPC1 thyroid papillary cancer cells and human embryonic kidney 293T (HEK293T) cells were obtained from the Chinese Science Institute (Shanghai, China). The TPC1 cells and Nthy-ori 3-1 cells were cultured in RPMI1640 medium (RPMI1640; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), penicillin (100 units/ml) and streptomycin (100  $\mu$ g/ml) (Enpromise, Hangzhou, China). The HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% FBS, penicillin (100 units/ml) and streptomycin (100  $\mu$ g/ml). Cells were incubated at 37°C in a humidified chamber supplemented with 5% CO<sub>2</sub>. Cells at approximately 90% confluence were split at 1:2 ratio every 2-3 days.

TPC1 cells ( $1 \times 10^5$ ) were added into each well of a 6-well plate and cultured with RPMI1640 medium without either serum or antibiotics. When TPC1 cell density reached 30-40%, cells were transfected with miR-181b inhibitor or inhibitor NC mimics purchased from Gene Pharma (Shanghai, China), using lipofectamine 2000 transfection reagents (Invitrogen, USA), according to the manufacturer's instructions. After 5-6 h of incubation, RPMI 1640 medium was replaced by RPMI1640 medium with 10% FBS. The sequences are as follows: miR-181b inhibitor, 5'-ACCCACCGACAGCAAUGAAUGUU-3'; InhibitorNC, 5'-CAGUACUUUUGUGUAGUACAA-3'.

#### *RNA extraction and quantitative reverse-transcription PCR (qRT-PCR)*

According to the manufacturer's instructions, expression levels of miR-181b were analyzed by using one-step qRT-PCR (EzOmics SYBR

qPCR kit); the miR-181b stem-loop primer, U6 primer and EzOmics SYBR qPCR kit were all purchased from Biomics Biotechnology Inc (Jiangsu, China). Real-time PCR was performed on a 7900HT fast real time-PCR instrument (Applied Biosystems, Singapore) using the following primers: miR-181b: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACACC-CCACCGAC-3' (stem-loop primer); 5'-CTAAGG-TGCATCTAGTGC-3' (sense); 5'-GTGCAGGGTCC-GAGGT-3' (antisense). U6: 5'-GTCCTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACAAAAT-ATGGAAC-3' (stem-loop primer); 5'-TGCGGGT-GCTCGCTTCGAGC-3' (sense); 5'-CCAGTGCAG-GGTCCGAGGT-3' (antisense).

RNA (100 ng) was added in a 25  $\mu$ l reaction mixture containing 12.5  $\mu$ l, 2 $\times$  Master mix, 0.5  $\mu$ l 50 $\times$  SYBR Green, 0.5  $\mu$ l reverse transcription primer (10  $\mu$ M), and 0.5  $\mu$ l sense and 0.5  $\mu$ l antisense primers (10  $\mu$ M) for miR-181b. One step PCR parameters for miRNA quantification were as follows: 37°C for 60 min for reverse transcription, 10 min at 95°C, followed by 40 cycles of 20 sec at 95°C, 30 sec at 62°C and 30 sec at 72°C. Ct values were collected at the end of the PCR. Each sample was tested in triplicate, and the relative quantification equation was used to calculate the relative expression.

For detection of CYLD mRNA expression, total RNA was isolated using TRIzol (Invitrogen, USA), and cDNA was generated by reverse transcription using the PrimeScript RT-PCR kit in accordance with the manufacturer's instructions (Takara, Japan). Real-time PCR was performed on a 7900HT Fast RT-PCR instrument using SYBR-Green and the following primers: CYLD, 5'-TCTATGG GGTAAATCCGTTGG-3' (sense), 5'-CAGCCTGCACACTCAT CTTC-3' (antisense);  $\beta$ -actin, 5'-CAGAG CCTCGCCTTGCC-3' (sense), 5'-GTCGCCACATAGGA ATC-3' (antisense). The PCR parameters for relative quantification were as follows: 2 min at 95°C, followed by 40 cycles of 45 sec at 57°C and 45 sec at 72°C. The relative expression was evaluated following the relative quantification equation,  $2^{-\Delta\Delta CT}$  [19]. Each sample was tested in triplicate.

#### Cell viability assay

Cells were plated in 96-well plates at  $2 \times 10^3$  cells per well 24 h post-transfection. MTT (20  $\mu$ l, 5 mg/ml) (Sigma, USA) was added to each well and cells were incubated for another 4 h at

37°C. The reaction was stopped by addition of 150  $\mu$ l DMSO (Sigma, USA) and after 10 min of low speed shaking (100 rpm) and incubation, optical density at 490 nm was determined on a microplate spectrophotometer. Each sample was tested with six replicates. All experiments were performed in biological triplicate.

#### Colony formation assay

TPC1 cells were seeded into six-well plates at 400 cells per well and grown for 14 days after 24 h of transfection with 100 nm miR-181b inhibitor or inhibitor NC. Cells were subsequently washed twice with phosphate-buffered saline (PBS). The colonies were fixed with 95% ethanol for 10 min, dried and stained with 0.1% crystal violet solution for 10 min, and the plate was washed three times with water. The number of colonies formed was counted in four different field visions and the mean value was calculated. Each treatment was performed in triplicate.

#### Apoptosis assay

TPC1 cells transfected with miR-181b inhibitor and negative control were seeded in six-well plates at  $10 \times 10^4$  cells per well and cultured for 48 h. Cells were subsequently double-stained with fluorescein (FITC)-conjugated Annexin V and propidium iodide (FITC-Annexin V/PI) (BD Biosciences, San Diego, CA, USA), and analyzed on a FACSCalibur flow cytometer (BD Biosciences) to determine rate of apoptosis.

#### Dual-luciferase reporter assay

293T cells were seeded in 12-well plates (BD, USA) and cultured until the cells reached 80-90% confluence. The CYLD 3'-UTR was cloned into the psiCHECK-2 vector, and co-transfected into cells together with miR-181b mimics or mimics NC (100 nm) using lipofectamine 2000, according to the manufacturer's instructions. Thirty hours after transfection, luciferase activity was measured using the Dual-Luciferase Reporter assay kit (Promega, USA). Briefly, the cells were washed twice with PBS, lysed with passive lysis buffer and incubated at room temperature for 15 min. The supernatants were collected and 20  $\mu$ l were added into 96-well plates. Firefly luciferase (FL) reporter was measured immediately after add-

## miR-181b and CYLD in thyroid cancer

**Table 1.** Differential expression of miRNAs between thyroid cancer tissues compared with para-cancer tissues

	MicroRNA	Fold change	P-value	
Upregulated	hsa-miR-146a	9.87	8.86e <sup>-4</sup>	
	hsa-miR-146b	11.70	3.12e <sup>-5</sup>	
	hsa-miR-15a	9.11	0.002	
	hsa-miR-155	6.46	0.015	
	hsa-miR-181a	9.16	1.58e <sup>-4</sup>	
	hsa-miR-181b	7.90	7.77e <sup>-4</sup>	
	hsa-miR-181d	5.22	0.006	
	hsa-miR-19a-3p	5.85	0.043	
	hsa-miR-221	8.16	3.11e <sup>-6</sup>	
	hsa-miR-222	8.33	1.49e <sup>-5</sup>	
	hsa-miR-34a	10.93	2.15e <sup>-8</sup>	
	hsa-miR-375	5.52	6.90e <sup>-5</sup>	
	hsa-miR-425	6.22	1.48e <sup>-4</sup>	
	hsa-miR-454-3p	3.76	0.021	
	hsa-miR-96	5.45	0.008	
	Downregulated	Hsa-miR-1	3.08	0.006
		Hsa-miR-101	3.93	0.003
Hsa-miR-10b-3p		4.43	8.30e <sup>-4</sup>	
Hsa-miR-193b-5p		3.73	2.84e <sup>-4</sup>	
Hsa-miR-30c-1-3p		3.04	0.007	
Hsa-miR-345-5p		3.85	0.001	
Hsa-miR-374a		4.76	3.24e <sup>-4</sup>	
Hsa-miR-363-3p		3.16	0.001	
Hsa-miR-370		3.04	0.004	
Hsa-miR-486-5p		3.57	7.90e <sup>-4</sup>	
Hsa-miR-513a-5p		2.59	0.010	
Hsa-miR-7	5.64	0.002		
Hsa-miR-718	3.22	0.020		

Compared with para-cancer tissues, in thyroid cancer tissues, a total of 158 miRNAs whose expression differences with a *P*-value of less than 0.05. Among them, almost 83 miRNAs expression up-regulated and 75 miRNAs down-regulated.

ing luciferase assay reagent II (LAR II). After quantifying the firefly luminescence, 100  $\mu$ l Stop & Glo<sup>®</sup> Reagent was added to each well to initiate the RL reporter and Renilla luminescence was then measured. The ratio of FL/RL was calculated to indicate the activity of the reporter.

### Western blot analysis

Cells were washed twice by cold PBS, and RIPA lysis buffer was added (1% Triton X-100, 50 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 20 mmol/L Iodoacetamide, 1 mmol/L phenylmeth-

anesulfonyl fluoride and 1% aprotinin). Cells were lysed on ice for 30 min, the cell lysate was collected into microtubes, and samples were centrifuged for 15 min at 12000 rpm at 4°C. Supernatants were collected and the protein concentrations were measured using the BCA Protein Assay Kit (Beyotime, Jiangsu, China). Protein samples (30  $\mu$ g) were denatured with 5 $\times$  sodium dodecyl sulfate (SDS) loading buffer (100 mmol/L Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerin, 200 mmol/L  $\beta$ -mercaptoethanol) at 95°C for 5 min. Protein samples were separated on a 10% SDS polyacrylamide gel electrophoresis and transferred onto 0.45  $\mu$ m nitrocellulose membranes (Beyotime). Following 60 min of blocking with 5% fat-free milk, membranes were incubated with CYLD antibody (1:1000, Proteintech, USA) and  $\beta$ -actin antibody (1:1000, Epitomics) overnight at 4°C. Blots were washed and incubated for 1 h with the anti-rabbit secondary antibody (1:1000, Epitomics). After three washes with PBST, immunoreactive protein bands were detected with an Odyssey scanning system (Li-Cor, Lincoln, NE, USA).

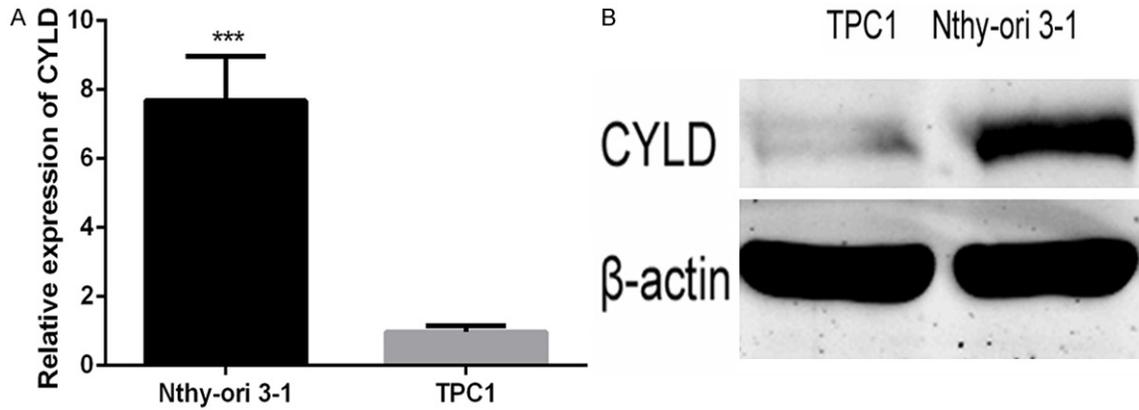
### Statistical analysis

GraphPad Prism version 6.0 (GraphPad, San Diego, CA, USA) was used for all statistical analyses. All data are expressed as mean  $\pm$  SEM. Differences between groups were analyzed by a 2-tailed Student's paired *t*-test for single comparisons and by one-way ANOVA with LSD post-hoc test for multiple comparisons. Bonferroni's correction was used to adjust for multiple comparisons. A *P* value < 0.05 was considered to be statistically significant.

## Results

### MiR-181b expression is increased in thyroid cancer

MiRNA microarray was performed to analyze the miRNA expression between thyroid cancer tissues and para-cancer tissues. We found that almost 83 miRNAs expression were up-regulated and 75 miRNAs down-regulated that had a *P*-value less than 0.05. We also found the expression of miR-181b was significantly higher in cancer tissues than para-cancer tissues, nearly 7.90 high fold change, as showed in



**Figure 1.** The CYLD expression is significantly higher in TPC1 cells compared with the levels in the Nthy-ori 3-1 normal thyroid cells both in mRNA and protein levels (\*\* $P < 0.001$ ).

**Table 1**, which was consistent with the finding of Pallante et al [12].

*CYLD expression is decreased in TPC1 thyroid cancer cells*

We examined the CYLD expression in TPC1 cancer cells and in Nthy-ori 3-1 of normal thyroid cells in mRNA and protein levels. As shown in **Figure 1**, CYLD is significantly lower in TPC1 cells compared with the levels in the Nthy-ori 3-1 normal thyroid cells ( $P < 0.001$ ). These results indicated that CYLD is significantly downregulated in TPC1 thyroid cancer cells.

*Downregulation of miR-181b in TPC1 cells inhibits cell proliferation and colony formation ability*

The relative expression of miR-181b were measured by qRT-PCR 30 h after TPC1 cells were transfected with 100 nm miR-181b inhibitor and NC control. The relative expression of miR-181b was significantly decreased in group of miR-181b inhibitor compared with the group of NC control (**Figure 2A**). The viability of cells transfected with 100 nm miR-181b inhibitor was measured and compared with that of NC at 24, 48, 72 and 96 h post-transfection. We found that the viability of miR-181b inhibitor groups was consistently significantly lower than that of NC groups in a time-dependent manner (**Figure 2B**). As shown in **Figure 2C**, the miR-181b inhibitor group exhibited fewer colonies than the

NC groups as determined by the colony formation assay. These results suggest that down-

regulation of miR-181b suppresses the proliferation and colony formation ability of TPC1 cells.

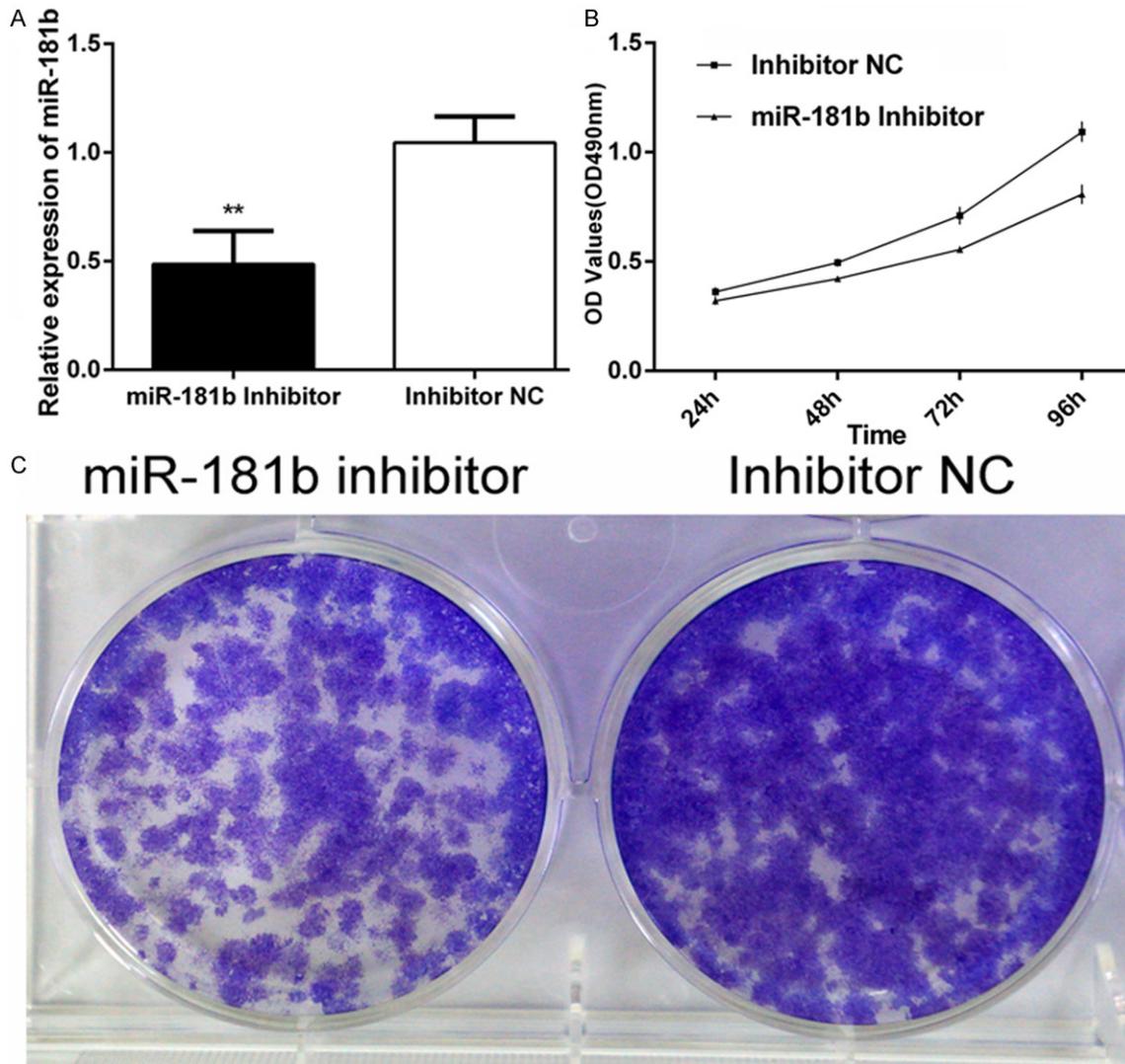
*Downregulation of miR-181b promotes the apoptosis of TPC1 cells of thyroid cancer*

To examine whether downregulation of miR-181b levels facilitates the apoptosis of TPC1 cells, TPC1 cells were transfected with 100 nmol/l of miR-181b inhibitor for 48 h. Flow cytometry data (**Figure 3**) indicated that the decreased expression of miR-181b induced early apoptosis, compared to the NC control groups, and the percentage of early apoptotic cancer cells of the miR-181b inhibitor treatment groups was markedly increased (treatment groups:  $7.64 \pm 0.06\%$ ; NC groups:  $2.03 \pm 0.76\%$ ), which shows that downregulation of miR-181b can promote apoptosis in TPC1 cells of thyroid cancer in vitro ( $p < 0.01$ ,  $n = 3$ ).

*CYLD is a direct target of miR-181b*

We searched for potential targets of miR-181b using several online databases, including targetscan, miRanda, miRBase and all these databases indicated that the

CYLD mRNA contained miR-181b binding sites. **Figure 4A** shows the potential binding sites of miR-181b to CYLD 3'-UTR according to prediction in the website of targetscan, and several papers had already reported that CYLD is a direct target of miR-181b in breast cancer cells [18] and pancreatic cancer cells [20]. To examine and confirm the possibility that miR-181b targets CYLD, we constructed a psiCHECK-2/



**Figure 2.** Down-regulation of miR-181b expression inhibited the growth of TPC1 cells: A. TPC1 cells were transfected with miR-181b inhibitor or inhibitor-NC at 100 nmol/l for 36 h and miR-181b inhibitor could inhibit the expression of miR-181b significantly, measured by qRT-PCR (\*\* $P < 0.01$ ); B. The proliferation of TPC1 cells transfected with miR-181b inhibitor was inhibited in a time-dependent manner compared with the inhibitor NC group, measured by MTT cell proliferation assays. Results represent mean  $\pm$  SD (\* $P < 0.05$ ). C. Colony formation assays showed Crystal Violet staining of the miR-181b inhibitor transfected group and inhibitor NC transfected group.

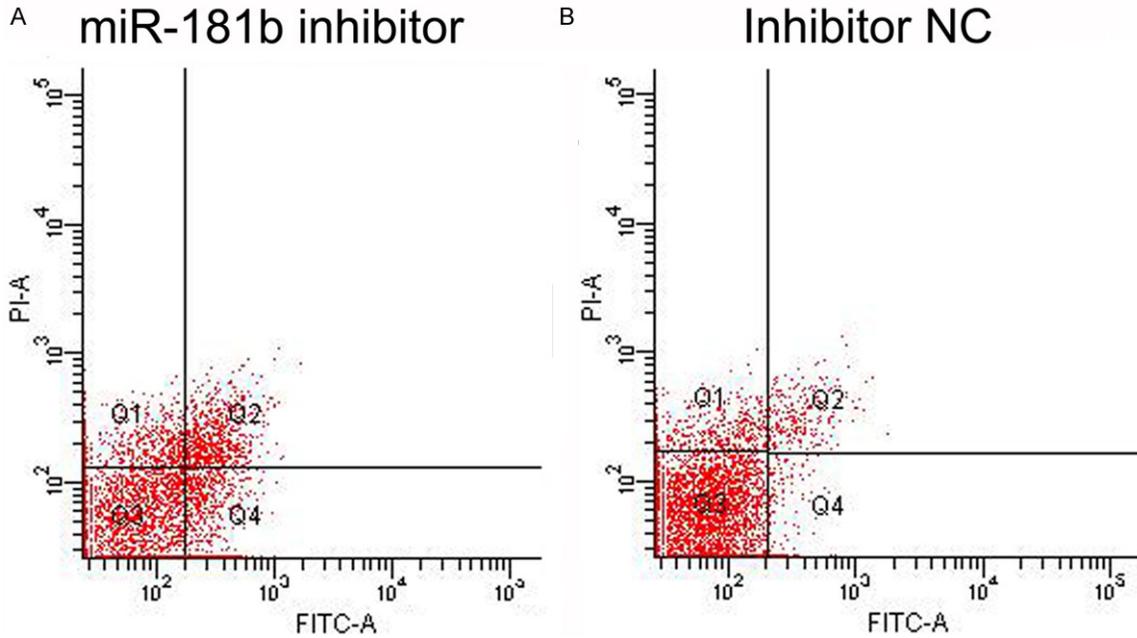
CYLD 3'-UTR vector, and cells were transfected with either miR-181b or NC mimics in HEK 293T cells, along with psiCHECK-2/CYLD 3'-UTR. The luciferase activity was significantly decreased following co-transfection of psiCHECK-2/CYLD 3'-UTR with miR-181b, compared with the miR-NC group ( $P < 0.05$ ) (Figure 4B). These results show that miR-181b could directly interact with the CYLD 3'-UTR in the psiCHECK-2 reporter plasmid.

Furthermore, RT-PCR analysis (Figure 4C) and western blot analysis (Figure 4D) showed that

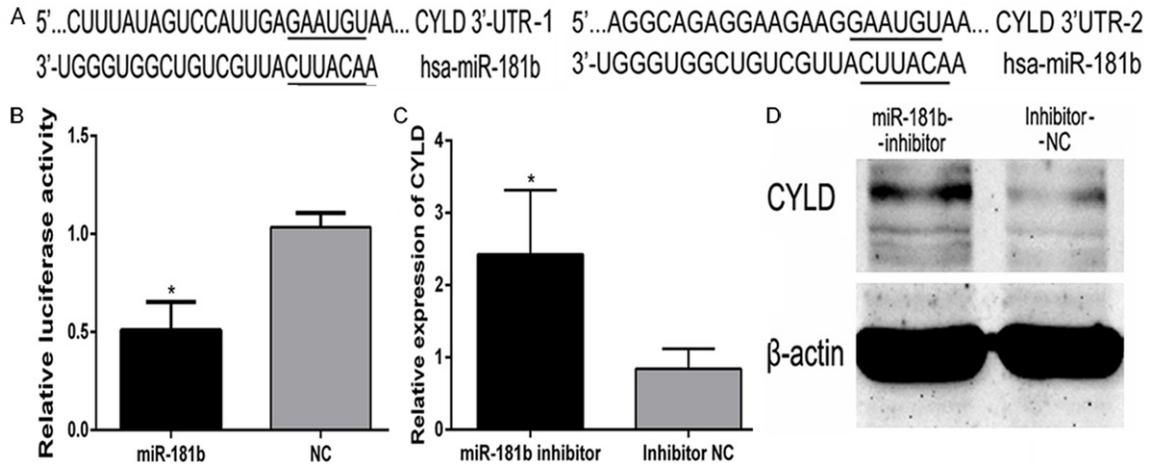
when miR-181b expression was inhibited, CYLD mRNA and protein levels both were higher in the miR-181b inhibitor group compared with the NC group. Taken together, these results indicate that miR-181b could directly bind to the CYLD 3'-UTR region to regulate CYLD in TPC1 cells.

#### Discussion

A lot of miRNAs have been identified and annotated and many evidences indicate that miRNAs are differentially expressed between nor-



**Figure 3.** Effects of transfection with miR-181b inhibitor or inhibitor NC of apoptosis on TPC1 cells. The early apoptosis of TPC1 cells in the miR-181b groups was  $7.64 \pm 0.06\%$ , which was statistically different from  $2.03 \pm 0.76\%$  of the Inhibitor NC groups ( $n = 3, P < 0.01$ ).



**Figure 4.** CYLD is a direct target of miR-181b: A. Diagram of CYLD 3'-UTR-containing reporter constructs; B. Luciferase reporter assay in 293T cells transfected with reporter vectors containing CYLD 3'-UTR. Relative luciferase expression was normalized to Renilla activity; C, D. RT-PCR and Western blot analysis showed the response to down-regulation of miR-181b by miR-181b inhibitor from mRNA and protein levels of CYLD.

mal and tumor tissues, suggesting that miRNA play an important role in tumorigenesis [21, 22]. In this study, we performed MicroRNA array analysis to investigate the expression pattern of miR-181b in primary human thyroid cancer. MiR-181b expression was significantly increased in thyroid cancer specimens compared with para-cancer tissues, which was similar

with some previous [12, 13]. Recent studies demonstrate that the NF-κB pathway controls proliferative and anti-apoptotic signalling pathways in thyroid cancer cells [16]. As described before, CYLD, a K63-specific deubiquitinase, has been demonstrated to negatively regulates NF-κB activity, inhibit the NF-κB signal transmission by specifically deconjugating the K63-

linked polyubiquitin chains from multiple intermediaries of the NF- $\kappa$ B signaling pathway [17, 18].

In breast cancer, there is an evidence showing that, CYLD, as a negative regulator of NF- $\kappa$ B signaling, was significantly downregulated in breast cancer tissues, and overexpression of CYLD, inhibited the proliferation and apoptosis in breast cancer cells [23].

In our experiments, we found that the expression of CYLD in TPC1 thyroid cancer cells was significantly lower when compared with the normal thyroid cell lines (Nthy-ori 3-1). According to the results of our MicroRNA array analysis, to assess the role of miR-181b in thyroid cancer, we investigated the loss of function effects of miR-181b on TPC1 cells of thyroid cancer. The present study demonstrated that targeted knockdown of miR-181b expression by inhibitors in TPC1 cells led to significant inhibition of cellular proliferation, and promoted apoptosis. Previous studies showed that miR-181b has played a role in regulating cellular growth, migration, invasion and apoptosis in several kinds of tumors, such as gastric adenocarcinomas [8], chronic lymphocytic leukemia [9], ovarian cancer [10] and so on. Taken together, these results indicate that deregulated expression of miR-181b may function as an oncogene in multiple cancers.

Our results have shown that downregulation of miR-181b in TPC1 cells of thyroid cancer significantly influences cell proliferation, and apoptosis. CYLD, as a directed target of miR-181b, has been reported in breast cancer and pancreatic cancer [18, 20]. We also searched for potential targets of miR-181b using several online databases, including targetsScan, miRanda, miR-Base, and all databases indicated that the CYLD mRNA contained miR-181b binding sites. To test whether CYLD is a real target of miR-181b in thyroid cancer, we constructed the psiCHECK-2/CYLD 3'-UTR plasmid, which contains the 3'-UTR of CYLD. Through dual-luciferase assays, we confirmed the CYLD 3'-UTR as a direct target of miR-181b in HEK 293T cells. Additionally, we found that endogenous CYLD expression, both at the mRNA and protein levels, was increased in TPC1 cells transfected with miR-181b inhibitors. In conclusion, we demonstrated in this study that downregulation of miR-181b inhibited the proliferation and pro-

moted apoptosis in thyroid cancer TPC1 cells, and that this effect was mediated, at least partly, by CYLD. These results suggest that downregulation of miR-181b level may be a potential new target for the treatment of thyroid cancer.

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### Disclosure of conflict of interest

None.

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